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ORIGINAL PAPER

Synergistic Bcl-2 inhibition by ABT-737 and cyclosporine A

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Abstract Survival of lymphocytes and melanocyte stem cells critically depends on B cell lymphoma 2 (Bcl-2). In T lymphocytes, a basal calcineurin activity maintains Bcl-2 expression in naïve cells, and the activation of the calcineurin pathway orchestrates the regulation of the intrinsic apoptosis pathway after antigen recognition. Therefore, calcineurin inhibitors might potentiate the pro-apoptotic effect of pharmacological Bcl-2 inhibitors on lymphatic cells. In vitro, a reduced Bcl-2 expression in lymphocytes exposed to calcineurin inhibitors increased their sensitivity to the small molecule Bcl-2 inhibitor ABT-737. This correlated with an augmented pro-apoptotic activity of ABT-737 on lymphocytes in combination with cyclosporine A in naïve mice in vivo. Interestingly, similar processes were observed in melanocytes. ABT-737 induced a fur depigmentation at the site of injection, and this effect was expanded to a generalized depigmentation in combination with cyclosporine A. Thus, inhibiting calcineurin increases the pro-apoptotic potency of ABT-737 in cells depending on Bcl-2 for survival. The increased efficacy of Bcl-2 inhibitors in combination with cyclosporine A might be relevant to exploit their anti-neoplastic and immuno-modulatory properties.

Keywords Bcl-2 · ABT-737 · Calcineurin · Cyclosporine A · Lymphocyte · Melanocyte

Laurence Feldmeyer and Thomas Fehr contributed equally to this work.

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Introduction

Bcl-2 (B cell lymphoma 2) is the prototype of an evolutionary conserved family of proteins that regulate the mitochondrial apoptosis pathway [1, 2]. Aside from its role as a proto-oncogene [3], Bcl-2 has a fundamental function in development and in the regulation of cell survival in different tissues [4]. This is well demonstrated by the phenotype of Bcl-2 deficient mice, characterized by accelerated lymphoid apoptosis, polycystic kidneys and accelerated hair depigmentation [4].

The advent of selective Bcl-2 inhibitors offers unexplored opportunities for a pharmacological interaction with these mechanisms. Because of their limited off-target effect the small molecule BH3-mimetics ABT-737 [5] and ABT-263 (Navitoclax) are of notable interest [6]. These rationally designed BH3-mimetics initiate the mitochondrial apoptosis cascade by inhibition of the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-XL and Bcl-w [5], induced apoptosis in several tumor models [7] and represent a promising class of anti-neoplastic agents [8, 9]. Moreover, in consideration of the multiple physiological functions of Bcl-

2, the same agents might find clinical applications in other settings. In particular, due to the critical role of the intrinsic apoptosis system in the immune system, Bcl-2 inhibitors displayed an immuno-modulatory effect and inhibited autoimmunity [10, 11] and allograft rejection [12, 13].

Lymphocytes tightly regulate the expression of pro- and anti-apoptotic members of the Bcl-2 family during different stages of differentiation. Bcl-2 is crucial for survival of naïve T cells [14], whereas other Bcl-2 family members are critical in hematopoietic stem cells, in thymocytes, in activated T cells and in memory cells [15]. The dynamic regulation of different Bcl-2 protein family members (not all of them efficiently inhibited by ABT-737) determined the selectivity profile of ABT-737 on different lymphocyte subpopulations and eventually its immuno-modulatory properties [16]. Treatment with classical immunosuppressive drugs, such as calcineurin inhibitors, had a marked impact on the regulation of the intrinsic apoptosis pathway in T cells after antigen recognition and significantly increased the immunosuppressive effect of ABT-737 [13]. On the other hand, calcineurin-deficient mice expressed a lower level of Bcl-2 in naïve T cells and were characterized by a moderate lymphopenia, indicating that Bcl-2 expression in naïve T cells also depends on the activity of the calcineurin-NFAT pathway [17]. A similar phenomenon was observed in T cells isolated from bronchoalveolar lavage fluid in asthmatic patients treated with cyclosporine A (CsA) [18].

In this study we investigated the effect of a blockade of the calcineurin/NFAT pathway on the pro-apoptotic potency of ABT-737 in adult mice. Calcineurin inhibitors reduced the expression of Bcl-2 in naïve lymphocytes and increased their sensitivity to ABT-737 in vitro and in vivo. Interestingly, this synergistic effect was not limited to lymphatic cells and similarly influenced platelets and melanocytes. As a result, treatment with ABT-737 and CsA partially reproduced the phenotype of Bcl-2 deficient mice, as shown by lymphocyte depletion and hair depigmentation, but not renal cyst formation.

Methods

Mice

C57BL/6 (B6, H-2^b) and Bim knock-out mice (Bim−/−, H-2^b) mice were housed in specific pathogen-free conditions at the University of Zürich. Bim−/− mice were kindly provided by Andreas Strasser [19]. All experiments were performed with 6–8 weeks old mice and according to protocols approved by the legal authority (Veterinary Office of the Canton of Zürich, Switzerland). Skin transplantation was performed using full thickness back skin (about 0.5–1.0 cm^2) as previously described [13].

Reagents and drugs

ABT-737 was provided by Abbott Bioresearch (Worcester, USA): for in vitro experiments ABT-737 was dissolved in DMSO at a concentration of 5 mM and then diluted in culture medium. For in vivo applications ABT-737 was dissolved in polyethylene glycol, tween 80, dextrose solution and DMSO and injected intra-peritoneally (i.p.). CsA was purchased from Enzo Biochem (Farmingdale, New York, USA), dissolved in ethanol and cremaphor EL (Sigma-Aldrich, Buchs, Switzerland), then diluted in PBS and injected subcutaneously (s.c.) at 10 mg/kg. Tacrolimus (FK506) was purchased from Enzo-Biochem, the NFAT-inhibitor VIVIT-R from Calbiochem (Merck, Darmstadt, Germany).

Cell culture

Splenocytes were cultured in RPMI medium containing 10 % fetal bovine serum, penicillin 100 U/mL, streptomycin 100 µg/mL, 2-mercaptoethanol 50 µM. CsA, Tac and VIVIT-R were added to the medium at the beginning of the culture. In some experiments, after 48 h of culture ABT-737 was added and 12 h later cell viability was assessed by propidium iodide exclusion in FACS.

Fluorescence activated cell sorting (FACS) and blood analyses

FACS analyses were performed with a BD-FACSCanto (Becton–Dickinson, Basel, Switzerland). For surface FACS staining cells were incubated with the required antibodies in FACS buffer at 4 °C for 30 min. For Bcl-2 staining, after surface staining the cells were permealized by 10 min incubation in Permeabilization Wash Buffer (Biolegend, Uithoorn, Netherlands), then incubated with the anti-mouse Bcl-2 antibody for additional 30 min at room temperature, according to the manufacturer's protocol. Anti-mouse CD3-FITC, CD3-APC, CD4-PE, CD8-APC, CD11b-PE-Cy7, B220-FITC, B220-PE and propidium iodide (PI) were purchased from eBioscience (Frankfurt, Gernamy), anti-mouse CD25-PE/Cy7 from Biolegend (Uithoorn, Netherlands), anti-mouse Bcl-2-PE from Becton–Dickinson (Basel, Switzerland). Hematological analyses were performed in the laboratory of the Division of Hematology at the University Hospital Zürich with an ADVIA 2120 flow cytometer (Siemens, Eschborn, Germany).

Histology and immunohistochemistry

Skin and organs were removed, fixed overnight in 10 % buffered formalin and embedded in parraffin following routine protocols. Sections of 3 µM thickness were stained with periodic acid-Schiff (PAS) for routine evaluation.

Formalin-fixed, paraffin-embedded skin sections were deparaffinized and rehydrated, then stained with a mouse monoclonal antibody against S-100 (Abcam, ab4066, dilution 1:200) after blocking of the unspecific signal with a goat Fab anti-mouse IgG (Rockland, 810-1102, dilution 1:50). The tissue was then incubated with Dako REALTM Link, Biotinylated Secondary Antibodies, and with Dako REALTM Streptavidin Alkaline Phosphatase (AP). The reaction is visualized by a RED chromogen (Dako REALTM Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse Code K5005). Slides were counterstained with hematoxilin. Conventional kidney histology was performed as previously described [20].

Statistics

Student *t* test was used to compare values between groups as appropriate. Half maximal inhibitory concentration (IC50)-values were calculated using a log(inhibitor) versus response model. A linear regression analysis was performed in some experiments. $P < 0.05$ was considered significant. Graph Pad Prism Software Version 5.0 was used for calculations.

Results

CsA enhances sensitivity to ABT-737 and down-regulates Bcl-2 in naïve T cells in vitro

The effect of a combined Bcl-2 and calcineurin inhibition was first assessed on lymphocytes in vitro. Lymphocytes were isolated from the spleen of naïve 6–8 weeks old mice and cultured under different concentrations of CsA during 36 h and subsequently treated with ABT-737 for additional 12 h. FACS analysis for cell viability indicated that lower concentrations of ABT-737 were sufficient to induce apoptosis in naïve T cells pre-incubated with CsA in a concentration dependent manner (Fig. 1a). Since the balance between the anti-apoptotic Bcl-2 and the pro-apoptotic Bim determines survival of naïve T cells [14], the role of these two factors in our model was specifically investigated. The same in vitro experiment was repeated using splenocytes isolated from Bim−/− mice. According to previous reports [14], higher concentrations of ABT-737 were required to induce apoptosis in Bim−/− T cells. However, pre-incubation with CsA similarly increased the pro-apoptotic potency of ABT-737 on wild type (wt) and Bim−/− cells, suggesting that this effect is not dependent on Bim (Fig. 1b). On the other hand, we found that CsA reduced Bcl-2 expression in viable CD3 positive lymphocytes (Fig. 1c). The same effect was measured using the alternative calcineurin inhibitor FK506 (tacrolimus) and the NFAT inhibitor VIVIT-R, indicating that this was not an off-target effect of CsA and that the activity of the calcineurin-NFAT pathway regulates Bcl-2 expression in CD4 and CD8 T cells (Fig. 1d). Moreover, down-regulation of Bcl-2 by CsA correlated with a decreased viability of T cells in culture (Fig. 1e) and with the half maximal inhibitory concentration of ABT-737 (IC50) required to induce apoptosis in T cells. Thus, blocking the calcineurin/NFAT pathway reduced the expression of Bcl-2, and a synergistic Bcl-2 inhibition was obtained by a combination of calcineurin inhibitors and BH3-mimetics in vitro.

CsA potentiates the pro-apoptotic effect of ABT-737 on lymphohematopoietic cells in vivo

The synergistic effect of ABT-737 and CsA was further characterized in vivo. Eight weeks old B6 mice were injected with ABT-737 (50 mg/kg, i.p.) and/or CsA (10 mg/kg, s.c.) twice daily during 3 days and then euthanized for FACS analysis. According to previous reports [12, 13], ABT-737 markedly reduced the number of B and T cells and induced a moderate thrombocytopenia in blood (Table 1) and in the spleen (Table 2). CsA had a limited effect on hematological values as a single agent, but significantly potentiated the effect of ABT-737 not only on lymphocytes, but also on platelets, resulting in a severe lymphopenia and thrombocytopenia in the combination group. Among T cells, ABT-737 was more effective in depleting CD8 than CD4 T cells, and this effect was even more pronounced in combination with CsA (Fig. 2). As a consequence, more than 95 % of the CD8 T cells were depleted in blood and spleen in mice treated with CsA and ABT-737. Similar results were obtained in the lymph nodes (data not shown). In contrast, erythrocytes and neutrophil granulocytes, which are not affected by ABT-737 and CsA as single agents [13], were also not depleted in the combination group (Table 1). Thus, CsA potentiated the effect, but did not alter the selectivity profile of ABT-737 on lymphatic and hematopoietic cells and did not induce a generalized myelosuppression.

ABT-737 induces fur depigmentation in synergism with CsA

Apart from its fundamental role in lymphocytes, Bcl-2 controls survival of melanocyte stem cells [21]. In our in vivo experiments we observed, that ABT-737 also had an impact on hair pigmentation. To characterize this phenomenon, B6 mice were shaved and injected s.c. with a single dose of ABT-737. After hair re-growth an irreversible depigmentation of the fur was observed in the area treated with ABT-737, whereas no change was registered on the contralateral side that had been exposed to DMSO-containing vehicle (Fig. 3a). A similar process was

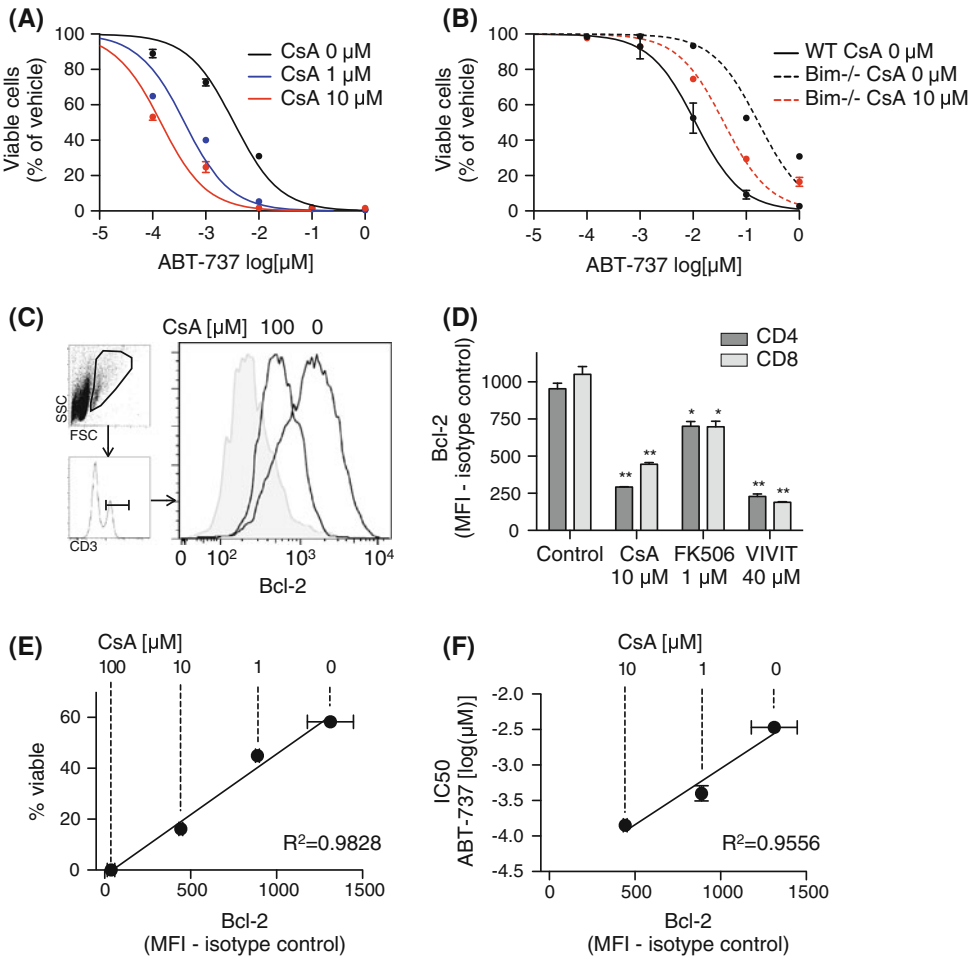

Fig. 1 Synergistic Bcl-2 inhibition in lymphocytes in vitro. **a** Splenocytes were incubated with CsA for 36 h, subsequently ABT-737 was added for additional 12 h of culture. Lower concentrations of ABT-737 were sufficient to induce apoptosis in cells pre-exposed to CsA [IC50 and 95 % confidence interval i*n* µM; CsA 0 µM: 0.0034 (0.0025–0.0045); CsA 1 µM: 0.0004 (0.0002–0.0006); CsA 10 µM: 0.0001 (0.00001–0.0002)]. **b** In the same experimental setting Bim−/− cells displayed a higher resistance to ABT-737 [IC50 0.1703 µM (0.0782–0.3710)] compared to wt cells [IC50 0.0111 µM (0.0082–0.015)] but their sensitivity to ABT-737 was also reduced after pre-incubation with CsA [IC50 after incubation with CsA 10 µM: 0.0380 (0.0236–0.0613]). **c**, **d** Inhibition of the calcineurin–NFAT pathway reduced the expression of Bcl-2 in naïve T cells. FACS analysis was performed after 48 h of culture in the presence of the calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506) or of the NFAT inhibitor VIVIT-R. To selectively investigate cells, which were still viable before the cell membrane permeabilitation for intracellular FACS-staining, only FSC high cells were considered for FACS analysis (s. gating strategy). A representative example for CD3 positive cells and CsA is shown in histograms in **c** (isotype control, CsA 100 and CsA 0 µM). A quantitative analysis for CD4 and CD8 T cells and the different drugs is presented in **d**. **e** The lower expression of Bcl-2 correlated with a diminished spontaneous viability of CD8 T cells, as measured by propidium iodide exclusion in FACS in parallel cultures not exposed to the permeabilitation process. **d** Similarly, sensitivity to ABT-737 as determined by the half maximal inhibitory concentration of ABT-737 for cell viability linearly correlated with the expression of Bcl-2. Statistical comparison with control; * $P < 0.05$, ** $P < 0.01$. Each point was calculated from duplicates and normalized with cultures exposed to vehicle; the data were confirmed in at least 2 independent experiments

observed after treatment with ABT-737 injected i.p. during 14 days at 50 mg/kg/day. Interestingly, i.p. application induced depigmentation of the abdominal skin only, whereas head, chest and back were spared. This particular pattern suggested that a direct effect of ABT-737 from the peritoneal cavity was responsible for this process (Fig. 3b). A similar fur depigmentation was observed in agouti mice (CBA background), confirming that this was not a strain specific effect (data not shown). In contrast, hair depigmentation was not observed in Bim−/− mice, indicating that this effect of ABT-737 was strictly dependent on the presence of an intact intrinsic apoptosis pathway and that Bim is a critical pro-apoptotic factor in melanocytes (Suppl. Fig. 1) [22]. Interestingly, the synergism with CsA influenced also this effect of ABT-737. Simultaneous treatment with ABT-737 (50 mg/kg/day, i.p.) and CsA

Table 1 Effect of ABT-737 in combination with cyclosporine A on blood cells

	Groups				*P* value		
	A Vehicle	B CsA	C ABT-737	D CsA + ABT-737	A vs. B	B vs. D	C vs. D
Erythrocytes [10^{12}/L]	9.21 ± 0.24	9.27 ± 0.20	9.60 ± 0.16	9.24 ± 0.51	0.66	0.89	0.28
Leukocytes [10^9/L]	5.67 ± 0.89	3.37 ± 0.52	1.65 ± 0.59	1.29 ± 0.27	0.001	<0.0001	0.27
CD3 [10^9/L]	1.33 ± 0.19	1.19 ± 0.26	0.42 ± 0.15	0.22 ± 0.05	0.37	<0.0001	0.03
CD4 [10^9/L]	0.74 ± 0.13	0.72 ± 0.17	0.31 ± 0.12	0.18 ± 0.04	0.81	0.0001	0.05
CD8 [10^9/L]	0.50 ± 0.07	0.40 ± 0.09	0.07 ± 0.02	0.02 ± 0.00	0.08	<0.0001	0.002
B220 [10^9/L]	3.72 ± 0.71	1.77 ± 0.29	0.66 ± 0.18	0.15 ± 0.07	0.0005	<0.0001	0.001
Neutrophils [10^9/L]	0.48 ± 0.17	0.31 ± 0.10	0.48 ± 0.14	0.62 ± 0.17	0.09	0.009	0.30
Platelets [10^9/L]	1184 ± 176.8	1214 ± 97.58	540.3 ± 11.85	120.6 ± 30.24	0.75	<0.0001	<0.0001

Treatment: 3 days, 2 injections/day, ABT-737 (2 × 50 mg/kg/day), cyclosporine A (CsA, 2 × 10 mg/kg/day), $n = 5$

Table 2 Effect of ABT-737 in combination with cyclosporine A on spleen cells

	Groups				*P* value		
	A Vehicle	B CsA	C ABT-737	D CsA + ABT-737	A vs. B	B vs. D	C vs. D
Cells [10^6/spleen]	145.6 ± 27.1	110.4 ± 16.0	61.0 ± 14.9	38.2 ± 10.2	0.04	<0.0001	0.02
CD3 [10^6/spleen]	39.1 ± 10.8	31.1 ± 4.2	12.8 ± 3.5	6.4 ± 1.0	0.16	<0.0001	0.004
CD4 [10^6/spleen]	25.6 ± 7.7	20.0 ± 2.7	9.4 ± 2.7	4.7 ± 0.7	0.16	<0.0001	0.005
CD8 [10^6/spleen]	9.6 ± 2.2	8.5 ± 1.4	1.7 ± 0.7	0.4 ± 0.1	0.36	<0.0001	0.003
B220 [10^6/spleen]	93.6 ± 14.7	70.4 ± 12.1	39.2 ± 9.7	22.5 ± 6.1	0.03	<0.0001	0.01

Treatment: 3 days, 2 injections/day, ABT-737 (2 × 50 mg/kg/day), cyclosporine A (CsA, 2 × 10 mg/kg/day), $n = 5$

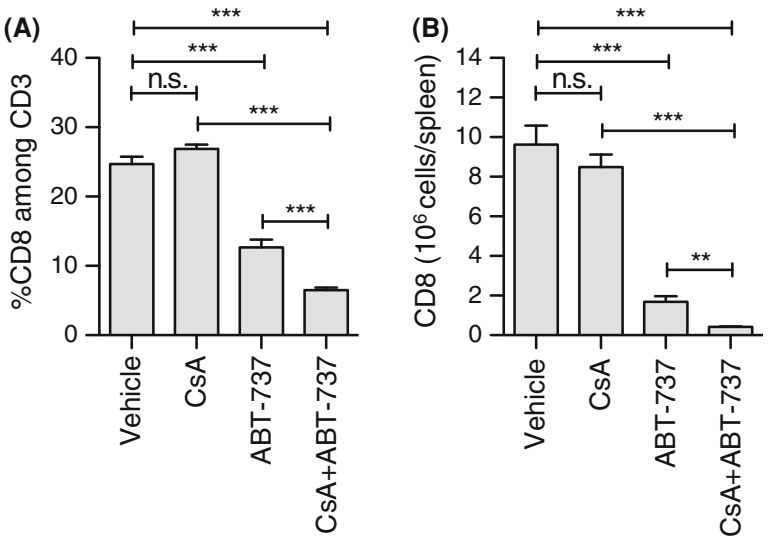

Fig. 2 Effect of ABT-737 and cyclosporine A on T cell subpopulations in vivo. Splenocytes were analyzed by FACS after a 3 day treatment with ABT-737 (2 × 50 mg/kg/day) or CsA (2 × 10 mg/kg/day). Among T cells (CD3+), we observed a more pronounced depletion in the CD8 T cell population in mice treated with ABT-737. This selectivity was even more pronounced in combination with CsA (**a**), resulting in an almost complete depletion of CD8 T cells in the spleen in the combination group (**b**). *** $P < 0.001$, n/group = 5

(10 mg/kg/day, s.c.) induced an irreversible systemic hair depigmentation, starting at the site of ABT-737 injection and progressing to a general hair involvement, whereas CsA alone did not show any effect (Fig. 3c).

In skin biopsies we observed a marked reduction in the number of melanocytes in the hair follicles in areas of depigmentated fur. This effect increased with time, being more accentuated 14 days after ABT-737 treatment in comparison to 7 and to 2 days (Fig. 4a). In biopsies taken 200 days after ABT-737 treatment we additionally observed a reduction in the number as well as in the size of hair follicles and a dysplastic hair shaft (Fig. 4b). Treatment with CsA alone had no effect on hair pigmentation, size or morphology. Notably, no inflammatory infiltrates were observed in skin biopsies. Moreover, transplantation of normal black B6 skin onto depigmentated B6 mice resulted in normal black skin pigmentation of the graft (Suppl. Fig. 2), indicating that depigmentation was the

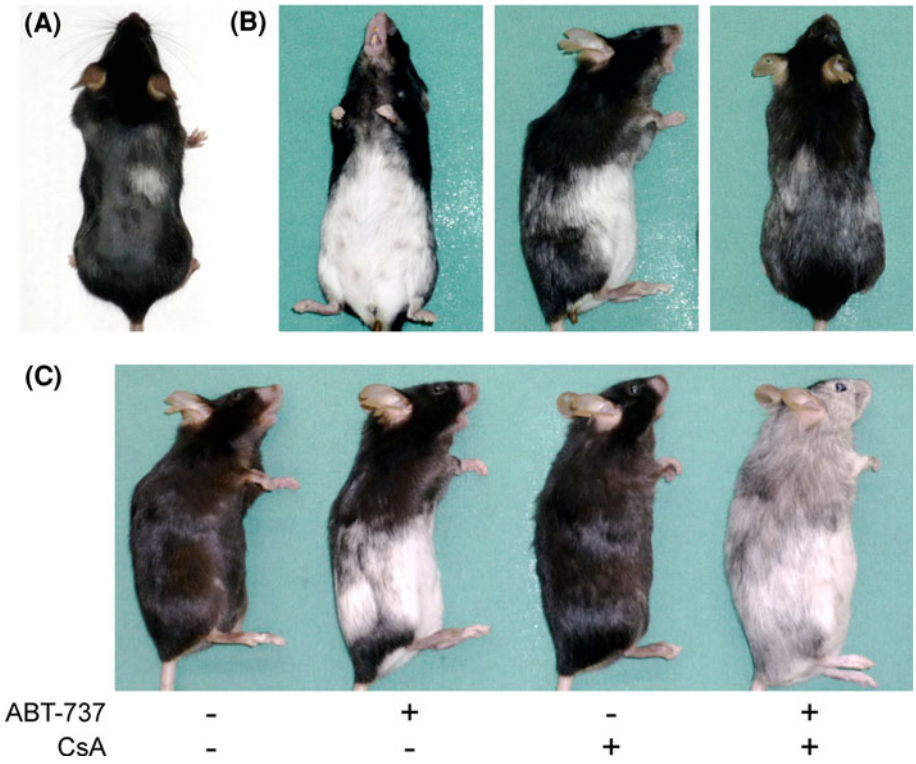

Fig. 3 Hair depigmentation after treatment with ABT-737 and CsA. **a** B6 mice were shaved and treated with a single s.c. injection of ABT-737 on the right side and of DMSO containing vehicle in the left side. After hair re-growth (3 weeks) we observed a localized hair depigmentation in correspondence of the ABT-737 injection. **b** After 14 daily i.p. injections with ABT-737 (50 mg/kg/day) a irreversible depigmentation of the abdominal fur was observed (photos: day 200 after treatment; one representative individual mouse in three different views is shown). **c** In combination with CsA, this effect of ABT-737 was markedly increased, resulting in a generalized irreversible fur depigmentation. The same phenomenon was observed in more than 50 mice in at least 5 independent experiments

effect of a short-term, direct effect of ABT-737 on melanocytes stem cells and not the result of an autoimmune (vitiligo-like) disease.

Absence of renal cyst formation in mice treated with ABT-737 and CsA

Additional effects of ABT-737 in combination with CsA were investigated by a histological analysis of the most important internal organs and—in consideration of the phenotype of Bcl-2 deficient mice—particularly of the kidney. ABT-737 did not increase the number of apoptotic cells in the kidney in the short term [13], but the effect of a long exposure to a Bcl-2 inhibitor on renal tissue is not known. In a first experiment, 6–8 weeks old B6 mice were treated with ABT-737 alone or in combination with CsA during 14 days and then observed for more than 200 days (Fig. 5a). In a second experiment, B6 mice were treated with the same regime during 50 consecutive days and then euthanized for histological investigations (Fig. 5b). In both cases the kidney tissue was normal, no cystic degeneration of the tubuli was observed and by conventional histology we did not detect any pathological alterations of the glomerular structure.

Discussion

This study was designed to investigate the effect of a combined Bcl-2 and calcineurin inhibition with a primary focus on lymphatic cells. Blocking the calcineurin/NFAT pathway induced a down-regulation of Bcl-2 in lymphocytes and increased their sensitivity to the Bcl-2 inhibitor ABT-737 in vitro and in vivo. Interestingly, this synergism was not limited to lymphocytes: mice treated with CsA and ABT-737 developed a more pronounced thrombocytopenia and also a generalized hair depigmentation as a result of melanocyte depletion. These results suggest that calcineurin inhibitors potentiate the pro-apoptotic effect of BH3-mimetics without altering their selectivity profile.

Previous studies characterized a complex interaction between the calcineurin/NFAT pathway and the regulation of apoptosis. Bcl-2 directly influences the activity of calcineurin by a direct molecular interaction, e.g. the nuclear translocation of NFAT is inhibited when calcineurin forms a complex with Bcl-2 [23]. On the other hand, the calcineurin/NFAT pathway itself is a key regulator of the apoptosis pathway [24]. Calcineurin directly dephosphorylates the anti-apoptotic Bcl-2 [25] and the pro-apoptotic Bad [26], and activation of the Ca^{2+}/calcineurin/NFAT pathway regulates the expression of Fas, Bcl-2A1 and many other pro- and anti-apoptotic factors in activated T cells [27, 28]. Apart from its essential role in T cell activation, calcineurin was also required for the homeostatic survival of T cells, as demonstrated by the Bcl-2 dependent, accelerated spontaneous lymphocyte apoptosis observed in calcineurin Aß deficient mice [17]. Moreover, CsA influences mitochondrial permeability in a

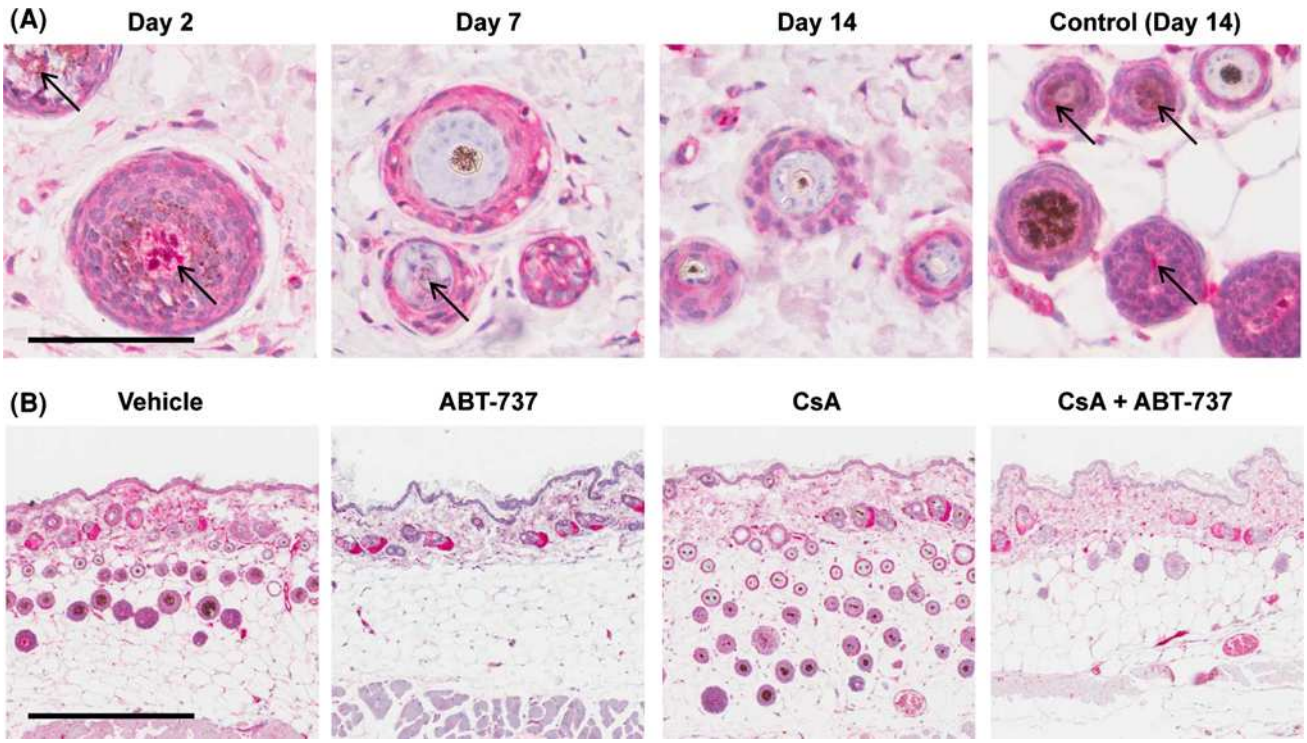

Fig. 4 Skin histology after ABT-737 treatment. **a** Skin biopsies were taken at day 2, 7 and 14 after s.c. injection of ABT-737 (50 mg/kg). Melanocytes (S-100 positive cells, *red, arrows*) and pigment (*brown*) progressively disappeared after exposure to ABT-737. *Bar* 100 μM, $n = 3$. **b** In the long term the hair depigmentation was accompanied by a reduction in the number and in the size of hair follicles, as shown in skin biopsies taken 200 days after a 2 weeks i.p. treatment with ABT-737 (50 mg/kg/day) and CsA (10 mg/kg/day). *Bar* 500 μM, $n = 3$ (Color figure online)

calcineurin-independent manner by interacting with cyclophilin D, and may therefore exert a direct pro- or anti-apoptotic effect [29, 30].

Because of the fundamental role of Bcl-2 in these highly interconnected and dynamically modulated processes, the advent of selective Bcl-2 inhibitors opens unexplored options for pharmacological combinations targeting the apoptosis pathway. We previously showed that a calcineurin-dependent up-regulation of Bcl-2A1 limits the pro-apoptotic potency of ABT-737 on T cells after antigen-recognition [16]. Here we show that the synergistic effect with CsA is not a prerogative of activated T cells. Because of the critical role of calcineurin for survival of naïve lymphocytes, CsA potentiated the pro-apoptotic effect of ABT-737 on B and T cells and induced a marked lymphopenia. The reduced Bcl-2 expression measured in lymphocytes exposed to inhibitors of the calcineurin-NFAT pathway represents a plausible explanation for this synergism, but—in consideration of the complexity of apoptosis regulation—additional molecular and pharmacological mechanisms are likely to be involved. In any case, this observation may be clinically relevant to increase the anti-neoplastic efficacy of ABT-737 on lymphatic tumors and to exploit the immuno-modulatory effect of ABT-737.

The moderate thrombocytopenia previously described after ABT-737 treatment was significantly increased in combination with CsA (Table 1) and may represent a relevant side effect in a clinical context. ABT-737 dependent thrombocytopenia is the result of Bcl-XL inhibition in platelets and megakaryocytes [31] and CsA enhanced platelet activation [32]. Further investigations are required to clarify the synergistic effect of ABT-737 and CsA in this particular setting.

Survival of melanocyte stem cells depends on Bcl-2. As a result, the physiological mechanism of hair graying is accelerated in Bcl-2 deficient mice [33]. The i.p. application of ABT-737 reproduced this phenotype by inducing melanocyte depletion and consequently hair depigmentation. To our knowledge, this effect of ABT-737 had never been described before. Moreover, we observed that it was dramatically increased in combination with CsA. ABT-737 as a single agent was sufficient to induce hair graying at the site of injection, but that a combination with CsA led to a generalized depigmentation, suggesting that CsA sensitized the melanocytic cells to the effect of ABT-737 in a similar way as we found in lymphocytes. This synergism may be relevant for the development of new melanoma therapies, particularly in consideration of previous studies showing an effect of ABT-737 on melanoma cells [34]. Moreover, the

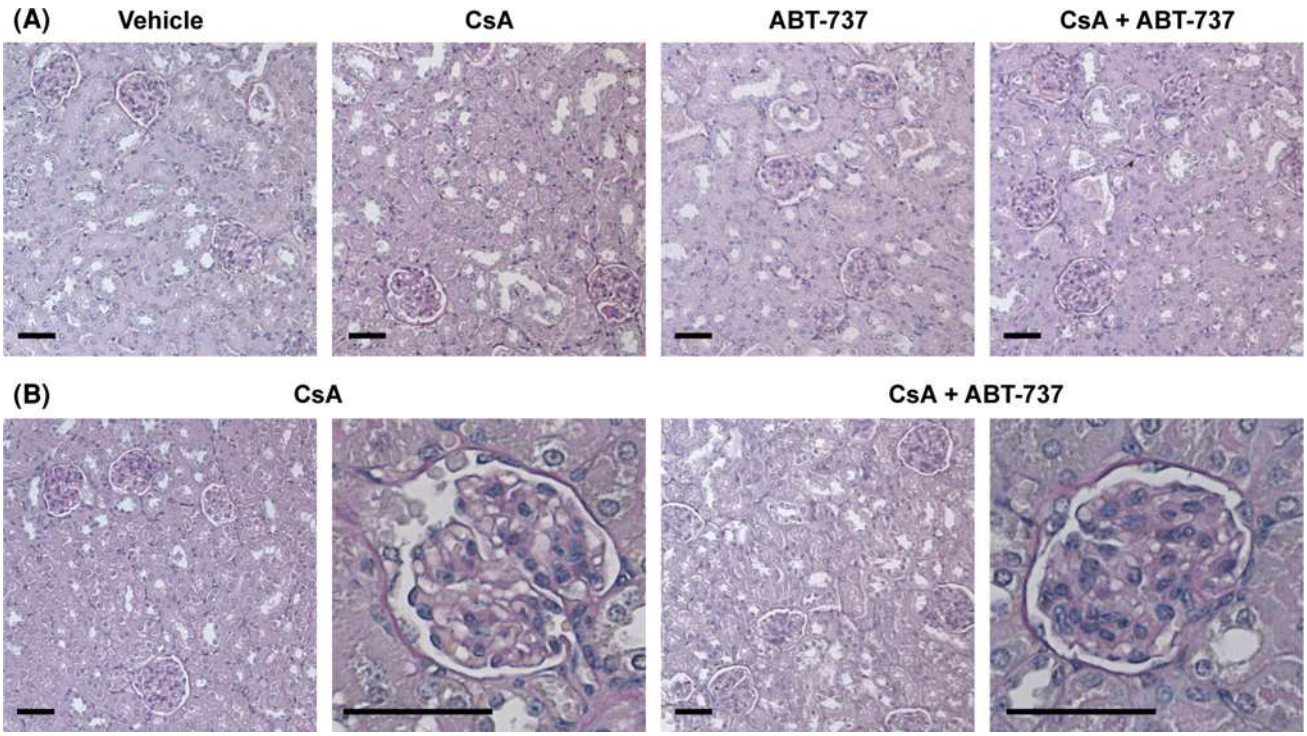

Fig. 5 Normal kidney structure after treatment with ABT-737 and CsA. **a** Daily exposure to ABT-737 (50 mg/kg/day) and CsA (10 mg/kg/day) during 14 days did no induce any long-term alteration of the renal structure (tissues taken 200 days after treatment). **b** Similarly, daily injections of ABT-737 (50 mg/kg/day) and CsA (10 mg/kg/day) during 50 days did not result in neither a polycystic degeneration of the renal tissue nor in any morphological glomerular alteration. *Bar* 50 μM

dysplastic alteration of the hair follicle observed in the long-term after ABT-737-induced melanocyte depletion may represent an innovative model to investigate the role of melanocyte stem cells in hair follicle biology.

Bcl-2 deficient mice are characterized by an abnormal renal development, manifest congenital kidney hypoplasia with polycystic degeneration and renal failure. Although a disturbed regulation of DNA-synthesis, apoptosis and cell-adhesive and migratory properties have been shown in Bcl-2 deficient renal tubular cells [35, 36], the exact pathogenesis of this disorder is not known. ABT-737 did not induce any alteration of the renal structure also after a long treatment and in combination with CsA, indicating that the role of Bcl-2 is presumably limited to a defined stadium during organogenesis [37].

In conclusion, calcineurin and Bcl-2 inhibitors displayed a potent synergistic effect that may be relevant to exploit the anti-neoplastic and immuno-modulatory effect of BH3-mimetics and to further investigate the role of Bcl-2 in lymphocyte, in the skin and in renal development.

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Conflict of interest We do not have any conflict of interest to declare.

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